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Executive Appendix

Articles

Lymphocyte Populations in Atherosclerotic Lesions of ApoE -/- and LDL Receptor -/- Mice

Decreasing Density With Disease Progression

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Abstract

Lymphocytes are prominent components of human atherosclerotic lesions, but their presence in murine models of disease has not been confirmed. Lymphocyte subpopulations have been identified in apoE -/- and LDL receptor -/- mice fed a cholesterol-enriched diet for up to 3 months. ApoE -/- mice had higher serum cholesterol concentrations than did LDL receptor -/- mice during most of the feeding period, primarily due to large increases in VLDL concentrations. Total area of atherosclerotic lesions was greater at all times in apoE -/- than LDL receptor -/- mice (lesion area after 3 months on cholesterol-enriched diet: apoE -/-, 993±193 and LDL receptor -/-, 560±131 $\mu\text{m}^2 \times 10^3$, mean±SEM, n=6 in each group). Lesions in apoE -/- mice contained larger macrophage-rich necrotic cores and more calcification than did those in LDL receptor -/- mice. Immunocytochemical analyses of tissue sections of ascending aortas performed with monoclonal antibodies to T and B lymphocytes and macrophages revealed that T lymphocytes immunoreactive for Thy 1.2, CD5, CD4, and CD8 were observed in lesions from both strains, but no B lymphocytes were detected. The density of Thy 1.2⁺ T lymphocytes in lesions was greatest at 1 month (apoE -/-, 98±23 and LDL receptor -/-, 201±40 lymphocytes/mm², n=6 in each group), decreasing in apoE -/- mice to 12±3 and in LDL receptor -/- mice to 51±20 lymphocytes/mm² at 3 months. The presence of T lymphocytes in murine atherosclerotic lesions makes these animals potentially useful for studying the involvement of the immune system in atherogenesis.

Key Words: atherosclerosis • murine model • T lymphocytes • immunohistochemistry

Cellular processes that occur during human atherogenesis may be examined by using animal

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models of atherosclerosis that simulate human disease. The PDAY study¹ established similarities in the evolution of atherosclerotic disease in humans and Watanabe heritable hyperlipidemic rabbits, cholesterol-fed rabbits,^{2,3} and rhesus monkeys.^{4,5} In addition, initial studies with cholesterol-fed C57BL/6J mice have led to the increasing use of mice in the study of events in atherogenesis.⁶ More recently, genetically modified mice deficient in either apoE⁷ or LDL receptors⁸ have become available. ApoE-deficient mice are grossly hypercholesterolemic and spontaneously develop atherosclerosis that has the morphological characteristics of human disease^{9,10,11}; disease development is accelerated by feeding these mice cholesterol-enriched diets.^{12,13} LDL receptor deficiency in mice produces only a mild increase in plasma cholesterol concentrations but imparts an increased responsiveness to cholesterol-enriched diets, leading to pronounced atherosclerotic lesion development.¹⁴

Atherosclerotic lesions are mostly made up of macrophages and smooth muscle cells, but there is increasing recognition of the presence of T lymphocytes.^{15,16,17,18} Both CD4⁺ and CD8⁺ T lymphocytes are present at all stages of development of human lesions.^{19,20} These T lymphocytes are activated, as judged from the presence of activation markers²¹ and expression of MHC class II antigens on adjacent smooth muscle cells.²² Expression of MHC class II is induced by the T lymphocyte-derived cytokine interferon gamma, which is detectable in lesions.^{23,24} T lymphocytes in atherosclerotic lesions are polyclonal in origin.²⁵ The full spectrum of antigens against which T lymphocytes are directed has not been elucidated, but it is known that oxidized LDL activates a small subset.²⁶ B lymphocytes are also found in human atherosclerotic lesions.^{15,27}

The role of the immune system in atherogenesis is controversial. Lesions that develop in cholesterol-fed rabbits contain T lymphocytes that may be active participants in lesion formation since immunosuppression results in enhancement of the atherogenic process.²⁸ The severity of atherosclerotic lesions is also increased in immune-suppressed²⁹ and MHC class I-deficient C57BL/6J mice³⁰ fed a cholesterol-enriched diet. However, in contrast to lesions in hypercholesterolemic rabbits, lymphocytes have not been detected in murine atherosclerotic lesions.³¹ Studies to define the role of the immune system in atherogenesis require an animal model in which T lymphocytes are present in lesions, as they are in human disease. We therefore used monoclonal antibodies to evaluate whether lymphocytes are present in atherosclerotic lesions of cholesterol-fed apoE^{-/-} and LDL receptor^{-/-} mice. Immunostaining for Thy 1.2, CD5, CD4, and CD8 was positive in atherosclerotic lesions in both strains of mice, although the density of T lymphocytes in each strain differed markedly. The presence of lymphocytes in atherosclerotic lesions of these mice makes them valuable for the study of the role of the immune system in atherogenesis.

Methods

Animals

LDL receptor^{-/-} and apoE^{-/-} mice (8 female and 10 male in each group) were obtained from Jackson Laboratories. Both strains of mice were originally generated as C57BL/6JxC129 hybrids,

and mice used in this study were backcrossed six generations into a C57BL/6J background. Mice were housed in specific pathogen-free rooms and fed a normal mouse laboratory diet (Ralston Purina) until they were 6 weeks of age, after which they were fed a diet containing 1.25% cholesterol, 0.5% cholic acid, and 15% fat (Harlan Teklad, catalogue No. 88051) for up to 3 months. All procedures were approved by the Washington University Animal Studies Committee.

Removal of Aortas and Blood Samples

Six mice of each strain were selected after 1, 2, and 3 months on a high-cholesterol diet. Nonfasting animals were anesthetized by metaphane inhalation (Pitman-Moore), bled retro-orbitally, and killed by cervical dislocation. Hearts were removed en bloc and placed in ice-cold Ringer's lactate, washed free of blood, and embedded and frozen in optimal cutting temperature compound (Tissue Tek). Sections of aorta (10 µm) were cut on a cryostat⁶ and placed on Probe-on-Plus microscope slides (Fisher Scientific). Serum was separated from whole blood by centrifugation and stored at 4°C.

Serum Cholesterol Concentrations and Lipoprotein Cholesterol Distribution

Serum concentrations of total cholesterol were measured by using an enzyme-based colorimetric assay (Wako Chemical Co). Lipoprotein cholesterol distributions were determined by fast-performance liquid chromatography of pooled serum samples from all six mice in each group after 3 months of feeding.³²

Histology and Immunocytochemistry

Frozen sections were fixed in acetone for 5 minutes. Macrophages were detected with anti-mouse monoclonal antibody MOMA-2 (rat IgG_{2b}, Serotec). All lymphocyte antibodies were initially screened for their ability to stain cells in splenic tissue (positive control). T lymphocytes were immunostained with monoclonal antibodies to murine CD5 (clone 57-7.3, rat IgG_{2a}K, Life Technologies), Thy 1.2 (clone 30-H12, rat IgG_{2b}, Collaborative Biomedical Products, and clone AT83A, rat IgM, Dr Osami Kanagawa, Washington University), CD8 (clone YTS 105.18, rat IgG_{2a}, Serotec), and CD4 (clone GK1.5, rat IgG, Dr Osami Kanagawa). Tissue sections were blocked with nonimmune rabbit serum. The secondary antibody was an affinity-purified, mouse serum-adsorbed, biotinylated rabbit anti-rat IgG (BA 4001, Vector Laboratories).

Immunocytochemical analysis was performed by using a Fisher MicroProbe system and Vectastain Elite ABC kits (Vector). Negative controls were obtained with isotype-matched irrelevant antibodies or no primary antibody. Immunoreactivity was visualized by using 3-amino-9-ethyl carbazole (Biomeda Corp), which forms a red precipitate. Accumulation of neutral lipid in lesions was visualized by staining with oil red O. Tissue sections were counterstained with aqueous hematoxylin (Biomeda).

Quantification of Lesion Areas and Numbers of T Lymphocytes

Consecutive 10-µm-thick aortic cross sections were cut, beginning at the most proximal part of the aortic sinus.⁶ Sections were placed consecutively on each of eight separate slides, after which

the ninth section was placed on the first slide, next to the first section, continuing for 48 sections. A single slide, upon which were six aortic cross sections from each mouse, was analyzed for lesion dimensions and for any given stain or immunostain. Total atherosclerotic lesion area and numbers of Thy 1.2⁺ lymphocytes were quantified by using an image-analysis system consisting of a Nikon Optiphot-2 microscope attached to a Javelin JE3462 high-resolution camera and a personal computer equipped with a Coreco-Oculus OC-TCX frame grabber and high-resolution monitor. Computerized color-image analysis was performed by using Image-Pro Plus software (Media Cybernetics). The area of each lesion in all six cross sections in every mouse was recorded, as was the total number of T lymphocytes determined by immunostaining for Thy 1.2. For each mouse studied, total atherosclerotic lesion area was calculated as the sum of the areas of all lesions in all six aortic cross sections on one slide. Thy 1.2-immunopositive lymphocytes were counted per section, and T-lymphocyte density was expressed as the number of lymphocytes per square millimeter of atherosclerotic lesion area.

Statistics

Differences in serum cholesterol concentrations, atherosclerotic lesion areas, and T-lymphocyte numbers were compared either by two-tailed Student's *t* test, or, if data failed to meet the requirements for use of this parametric test, by the Mann-Whitney rank-sum test. Data analyses were performed by using SigmaStat for Windows (Jandel Scientific).

Results

All animals tolerated the cholesterol-enriched diet without overt adverse affects. Total serum cholesterol concentrations before commencing the diet and at 1 and 2 months were higher in apoE -/- than LDL receptor -/- mice, but they did not differ significantly at 3 months (Fig 1E). Analyses of lipoprotein cholesterol distribution by size-exclusion fast-performance liquid chromatography demonstrated that regardless of diet, apoE -/- mice carried the major fraction of cholesterol in VLDL, while LDL receptor -/- mice carried cholesterol predominantly in an LDL-sized fraction.^{8 9 10 11}

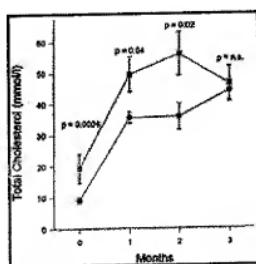


Figure 1. Line graph shows total serum cholesterol concentrations in apoE -/- and LDL receptor -/- mice. Cholesterol concentrations were measured by using enzymatic assays as described in "Methods." Points indicate means of six observations; bars, SEM; ■, apoE -/- mice; and •, LDL receptor -/- mice.

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Atherosclerotic lesions were characterized after 1, 2, and 3 months of cholesterol feeding for size, macrophage content, and lymphocyte number and distribution. The two strains of mice had atherosclerotic lesions with markedly different cellular architectures and areas. At all times, aortic atherosclerotic lesions of apoE -/- mice were larger than those in LDL receptor -/- mice (Fig 2). Lesions from the two types of mice were of similar cellular composition after 1 month of cholesterol feeding, composed predominantly of macrophages. By 3 months, lesions in apoE -/- mice had large cores of necrotic macrophages, a feature less abundant in LDL receptor -/- mice. Chondrocytes and early bone formation were readily discernible in all apoE -/- mice examined at 3 months, but in only one of six LDL receptor -/- mice. Bands of smooth muscle cells and extracellular matrix were present in apoE -/- but not LDL receptor -/- mice after 3 months (Fig 3).

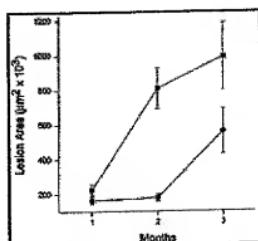


Figure 2. Line graph shows area of atherosclerotic lesions in apoE -/- and LDL receptor -/- mice after 1, 2, and 3 months on a cholesterol-enriched diet. Points indicate means of six observations; bars, SEM; ■, apoE -/- mice; and ●, LDL receptor -/- mice.

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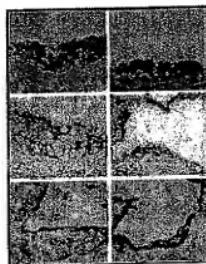


Figure 3. Photomicrographs show presence of macrophages and lipid deposits in murine atherosclerotic lesions. Aortic sections were immunostained for macrophages as described in "Methods." Macrophages were immunostained with MOMA-2 after 1 month of cholesterol feeding in (A) apoE -/- and (B) LDL receptor -/- mice. At 1 month, the two animal strains had lesions with similar morphological characteristics. After 3 months of cholesterol feeding, differences were observed in MOMA-2-immunostained macrophages: apoE -/- mice had necrotic macrophage core regions and macrophage accumulation under the endothelium separated by bands of nonstaining cells and matrix (C). In contrast, lesions from LDL receptor -/- mice immunostained uniformly for macrophages, and necrotic cores were uncommon (D).

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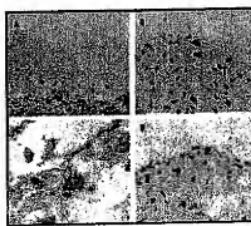
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Staining for neutral lipids with oil red O was patchy in apoE -/- mice after 3 months of cholesterol feeding (E) but relatively uniform in LDL receptor -/- mice (F) (original magnification x100 [A and B], x40 [C through F]).

T lymphocytes were detected by using antibodies to Thy 1.2, CD5, CD4, and CD8 (Table 1). Thy 1.2 and CD5 antigens are pan-T-cell markers, although CD5 is also present on a subset of B lymphocytes in serosal cavities. Immunostaining for Thy 1.2 and CD5 was observed in lesions from both strains. Furthermore, the distribution and number of cells exhibiting positive immunostaining was similar with both Thy 1.2 antibodies (Fig 4A-E) and the CD5 antibody (Fig 4B-E). Several monoclonal antibodies directed against T-lymphocyte antigen CD4 and an antibody to the B-lymphocyte marker CD45R were used to identify the subsets of lymphocytes present in mouse atherosclerotic lesions (Table 1). No B lymphocytes were observed in lesions, although the CD45R antibody produced excellent immunostaining of splenic tissue that was used as a control. Because only one of the anti-CD4 antibodies (GK1.5) resulted in appreciable splenic immunostaining, it was used to demonstrate the presence of CD4⁺ cells in atherosclerotic lesions (Fig 4C-E). In splenic tissue, CD4 immunostaining was less intense on positive cells than was Thy 1.2, CD5, and CD8 immunostaining. CD8⁺ cells were detected in the lesions of both strains (Fig 4D-E). The relatively low intensity of CD4⁺ subset immunostaining indicated that formal quantification may result in a misleading underestimate of cell numbers. Therefore, because robust immunostaining of T-lymphocyte subsets was not as consistently achieved as for Thy 1.2 antigen, no quantitative assessment of these subtypes was performed.

View this table: **Table 1.** Primary Antibodies Used to Detect Lymphocytes in

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Figure 4. Photomicrographs. A, T lymphocytes positive for Thy 1.2 in shoulder region of a lesion from an LDL receptor -/- mouse fed a cholesterol-enriched diet for 1 month. B, T lymphocytes positive for CD5 in an apo E -/- mouse fed a cholesterol-enriched diet for 3 months. C, T lymphocytes positive for CD4 in a lesion from an LDL receptor -/- mouse after 1 month of cholesterol feeding. D, T lymphocytes positive for CD8 in an atherosclerotic lesion from an LDL receptor -/- mouse fed a cholesterol-enriched diet for 2 months (original magnification x200 [A and B], x1000 [C], x400 [D]).

T lymphocytes, as determined by Thy 1.2 immunoreactivity, were present in atherosclerotic lesions at all intervals. The density of Thy 1.2⁺ T lymphocytes was greatest after only 1 month of cholesterol feeding in both strains of mice (Fig 5). At the intervals studied beyond 1 month, there was a significant reduction in lesion T-lymphocyte density, which was particularly sparse after 3 months in apoE -/- mice. At all intervals, lesions of LDL receptor -/- mice contained a greater density of Thy 1.2⁺ cells than did lesions of apoE -/- mice. In neither strain of mice was there a specific region in atherosclerotic lesions that preferentially accumulated T lymphocytes, as has been discerned in the human disease.¹⁵ The distribution of lymphocytes was patchy, with small foci of cells generally located beneath the endothelium and few cells near the media or in the lipid core. No T lymphocytes were detected in the media.

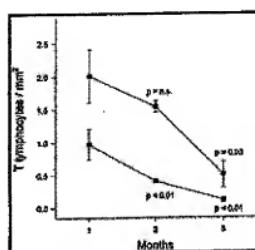


Figure 5. Line graph shows T-lymphocyte density of Thy 1.2⁺ cells in both strains of mice at 1, 2, and 3 months. Points indicate means of six observations; bars, SEM; ■, apoE -/- mice; and *, LDL receptor -/- mice. Statistical significance at 2 and 3 months (as determined by Mann-Whitney rank-sum test) is stated relative to the density at 1 month for each strain.

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Discussion

We observed striking differences in the dimensions and morphological characteristics of lesions in apoE -/- and LDL receptor -/- mice. Our observations of atherosclerotic lesions from apoE -/- mice are similar to earlier ones.^{10 11 12 13} Compared with LDL receptor -/- mice, lesions in apoE -/- mice were larger at all intervals studied and had a markedly increased number of chondrocytes and bands of smooth muscle cells. ApoE -/- mice had significantly increased concentrations of total serum cholesterol at most intervals, with most being in a VLDL fraction. Cholesterol-enriched VLDL has been demonstrated to promote cholesterol esterification in macrophages,^{33 34} which may be a factor in the formation of lesions of disparate morphology in apoE -/- and LDL receptor -/- mice, although this has not been proven.

The principal finding of this study is that Thy 1.2⁺, CD5⁺, CD4⁺, and CD8⁺ T lymphocytes are present in atherosclerotic lesions in cholesterol-fed apoE -/- and LDL receptor -/- mice. Thy 1.2 is a 112-amino acid glycoprotein present in varying amounts on the surface of neural and lymphoid cells, with expression depending on the state of differentiation.³⁵ In mice, Thy 1.2 is found on

mature T lymphocytes. CD5 is a monomeric 67-kD glycoprotein on all mature T lymphocytes, with higher expression on CD4⁺ than CD8⁺ cells.³⁷ CD5 also occurs on the B1a subset of B lymphocytes found in serosal cavities. CD5 functions as a tyrosine kinase substrate in association with the T-cell receptor ζ chain/CD3 and protein tyrosine kinases p56 ck and p59 fyn in T lymphocytes and may also act as an independent signaling molecule.³⁸

Previous immunohistochemical analyses of atherosclerotic lesions in several strains of mice, including the apoE -/- strain, have shown an absence of T lymphocytes.³¹ A possible explanation for this apparent contradiction is the interval at which lesions were studied. In the present study, lymphocyte density decreased with lesion maturity; particularly in apoE -/- mice, this cell type was sparse after 3 months of cholesterol feeding. The fact that Qiao et al³¹ studied lesions after cholesterol feeding of a longer duration than in the present study may explain the lack of detectable lymphocytes. In addition, in our study, several of the anti-CD4 antibodies tested resulted in weak and diffuse immunostaining of splenic tissue (Table II). Therefore, the difference between this and previous reports with regard to detection of lymphocytes might be partly attributed to differences in the affinity of antibodies used in immunohistochemical testing. However, while lymphocytes have not been reported in atherosclerotic lesions, CD4⁺, CD8⁺, and CD23⁺ (B lymphocytes) have been demonstrated in aortic fatty streaks of vasculitis-prone MRL/lpr mice.³⁹

In both apoE -/- and LDL receptor -/- mice, the density of T lymphocytes in lesions decreased as lesions matured. Signals responsible for recruitment of lymphocytes have not been defined, although one proposed mediator is the lysophospholipid formed by the oxidation of LDL.^{40 41} Early lymphocytic recruitment to atherosclerotic lesions occurred, but further development of lesions ensued without a proportional increase in T lymphocytes. The early recruitment of lymphocytes to atherosclerotic lesions has also been observed in cholesterol-fed rabbits^{42 43} and rats.⁴⁴ Lymphocyte residence time and trafficking within atherosclerotic lesions have not been defined but may be important parameters. Introduction of exogenous lymphocytes distinguishable on the basis of a genetically incorporated marker may assist in understanding the biology of lymphocytes within atherosclerotic lesions.

ApoE has been proposed as an endogenous regulator of the immune system, since it inhibits both monocyte⁴⁵ and T lymphocyte⁴⁶ proliferation. ApoE also inhibits interleukin-2-dependent T-cell proliferation, possibly by preventing transition from the G1_A phase of the cell cycle.⁴⁷ ApoE synthesis by macrophages varies according to the state of cell differentiation⁴⁸ and may be inhibited by interferon gamma⁴⁹ and stimulated by increasing intracellular cholesterol concentrations.⁵⁰ However, since apoE -/- mice develop severe atherosclerosis and inhibition of T lymphocytes enhances development of atherosclerosis,^{28 29 30} the physiological significance of the inhibitory effect of apoE on T lymphocytes in atherosclerotic lesions remains to be determined.

T lymphocytes are present in atherosclerotic lesions in apoE -/- and LDL receptor -/- mice,

making both strains useful for the study of immunologic factors affecting the development of atherosclerosis. In addition, we observed differences in morphological characteristics of lesions that could be due to altered lipoprotein metabolism or immunologic factors, both of which are likely to be targets of pharmacological intervention in the modulation of atherosclerotic disease.

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